

## Characterization of Hematopoietic Intracellular Protein Tyrosine Phosphatases: Description of a Phosphatase Containing an SH2 Domain and Another Enriched in Proline-, Glutamic Acid-, Serine-, and Threonine-Rich Sequences

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Protein tyrosine phosphatases (PTPases) are a family of enzymes important in cellular regulation. Characterization of two cDNAs encoding intracellular PTPases expressed primarily in hematopoietic tissues and cell lines has revealed proteins that are potential regulators of signal transduction. One of these, SHP (Src homology region 2 [SH2]-domain phosphatase), possesses two tandem SH2 domains at the amino terminus of the molecule. SH2 domains have previously been described in proteins implicated in signal transduction, and SHP may be one of a family of nonreceptor PTPases that can act as direct antagonists to the nonreceptor protein tyrosine kinases. The SH2 domains of SHP preferentially bind a 15,000-*M<sub>r</sub>* protein expressed by LSTRA cells. LSTRA cells were shown to express SHP protein by immunoprecipitation, thus demonstrating a potential physiological interaction. The other PTPase, PEP (proline-, glutamic acid-, serine-, and threonine-rich [PEST]-domain phosphatase), is distinguished by virtue of a large carboxy-terminal domain of approximately 500 amino acids that is rich in PEST residues. PEST sequences are found in proteins that are rapidly degraded. Both proteins have been expressed by *in vitro* transcription and translation and in bacterial expression systems, and both have been demonstrated to have PTPase activity. These two additional members of the PTPase family accentuate the variety of PTPase structures and indicate the potential diversity of function for intracellular tyrosine phosphatases.

Protein tyrosine phosphorylation is an important means by which cells control their behavior (12). However, the complete extent by which this regulatory modification influences cell function is only beginning to be understood. Both protein tyrosine kinases (PTKases) and protein tyrosine phosphatases (PTPases) are necessary to maintain the homeostatic balance required for tyrosine phosphorylation-mediated signaling. The kinase family is extensive and well characterized and is divided into two groups, intracellular and transmembrane (reviewed in reference 19). Many of the transmembrane PTKases are growth factor receptors in which soluble ligands bind to the external domain, resulting in activation of the cytoplasmic kinase domain and eventual entry into the cell cycle (reviewed in reference 63). The exact function of the intracellular PTKases is more obscure, although recently members of the *src* gene family, Lck and Fyn, have been shown to interact with CD4/CD8 and the T-cell antigen receptor, respectively, and are thus implicated in T-cell antigen receptor activation (48, 49, 62).

The characterization of the PTPase family is at a more rudimentary stage. However, studies show that this is also a multigene family, and, like the PTKase family, can be divided into intracellular and transmembrane members (reviewed in reference 10). PTPases are conserved in evolution, and forms have been identified in viruses (15), bacteria (17), yeast cells (16, 44), arthropods (55), nematodes (37), and mammalian species (6, 13, 22, 27, 31, 36, 54, 67). The most extensive characterization of PTPase sequences has come from a study of a protochordate (37). Analysis of 27 partial PTPase cDNA sequences from *Styela plicata* indicates that

there are subfamilies of PTPases, as judged by sequence similarity, and that some regions within the PTPase domain are conserved whereas others are very divergent.

Mammalian PTPases are divided into at least five subfamilies of transmembrane PTPases and several subfamilies of intracellular PTPases (10). Some subfamilies have several members, as indicated by the resemblance in structure of the non-PTPase domains and by the high degree of similarity of PTPase domain sequences. For example, within a subfamily, the PTPase domain shows approximately 70% sequence identity between individual members, compared with approximately 35% identity between different PTPase subfamilies. The structural diversity and differences in catalytic activity of the PTPases suggest that these enzymes participate in the control of a variety of biochemical pathways (56, 59, 60).

The mechanism by which protein tyrosine phosphorylation controls signaling has remained elusive. Recently, however, a specific functional role for phosphotyrosine has been elucidated. Activation of growth factor receptors leads to autophosphorylation and association with intracellular signaling proteins, implying that phosphotyrosine is a key component in the assembly of the complex (7, 9, 21, 23, 24, 32-34, 41). Among the molecules shown to engage directly growth factor receptors are members of the nonreceptor protein tyrosine kinase family, including c-Src, c-Yes, c-Fyn (29), the  $\gamma$  isoform of the phosphoinositide-specific phospholipase C (PLC- $\gamma$ ) (32-34, 41), p21<sup>ras</sup> GTPase-activating protein (GAP) (21, 23), and the phosphatidylinositol 3'-kinase-associated protein p85 (7, 21, 23). All of these cytoplasmic signaling molecules have disparate catalytic or binding domains but a common conserved noncatalytic region of ap-

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proximately 100 amino acids termed the Src homology region 2 (SH2) domain (reviewed in reference 25). The individual SH2 domains of GAP, PLC- $\gamma$ , p60<sup>src</sup>, and p120<sup>abl</sup> have been expressed and bind to phosphotyrosine-containing proteins, including activated growth factor receptors (1, 39, 42). In addition, the avian p47<sup>gag-crk</sup> viral transforming protein binds to phosphotyrosine-containing proteins via an SH2 domain (35, 38).

To understand how PTPases regulate leukocyte behavior, we have pursued the identification of PTPases expressed by hematopoietic cells. In this report, we describe the characterization of two additional PTPases expressed primarily by hematopoietic tissues. One of these PTPases, SHP (SH2-domain phosphatase), contains an amino-terminal domain with two SH2 domains and is a candidate for modulating SH2 domain-mediated signaling. The other PTPase, PEP (proline-, glutamic acid-, serine-, and threonine-rich [PEST]-domain phosphatase), contains an additional domain rich in sequences indicative of a short half-life.

## MATERIALS AND METHODS

### Isolation of PCR fragments encoding PTPase domains.

Methods used to isolate mouse polymerase chain reaction (PCR) fragments were essentially as described for the isolation of PTPase-encoding domains of *S. plicata* (37). Two micrograms of total RNA from either mouse brain or spleen was hybridized with 200 ng of a degenerate oligonucleotide pool B ( $n = 512$ ) derived from a compilation of PTPase sequences that in human CD45 is HCSAGVGR and consisted of 5'-CGCCC(A/G)A(T/C)(T/C/A/G)CC(T/C/A/G)GC(T/C/A/G)CT(A/G)CAGTG-3'. cDNA synthesis was initiated by the addition of 200 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla.) in a buffer containing 50 mM KCl, 20 mM MgCl<sub>2</sub>, 0.1 mg of bovine serum albumin (BSA) per ml, 1 mM deoxynucleoside triphosphates, and 20 U of RNasin (Promega, Madison, Wis.) and placed for 1 h at 42°C. The reaction mixture was heated at 95°C for 5 min and quickly chilled on ice prior to PCR. cDNAs for PTPase domains were amplified by adding 2  $\mu$ l of the reverse transcriptase reaction mixture to a standard PCR buffer in the presence of 200 ng of the same oligonucleotide pool B and oligonucleotide pool A ( $n = 576$ ) derived from the compilation of PTPase sequences that in human CD45 is DFWRMIWE and consisted of 5'-A(T/C)TT(T/C)TGG(C/A)(T/A/G)(T/G)ATG(A/G)T(T/C/A)TGG(C/G)A-3'. The PCR temperature settings for 30 cycles were 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min, using a Coy Tempcyler (Ann Arbor, Mich.). Fragments of 380 bp were isolated from a low-melting-point agarose gel and subcloned by *Eco*RI linker addition into the Bluescript KS vector (Stratagene, La Jolla, Calif.). Colonies were screened at a low stringency (35% formamide, 42°C) with a cocktail of 15 different <sup>32</sup>P-labeled PTPase subdomain cDNAs that had been previously isolated from the protochordate *S. plicata*. Positive colonies were picked and sequenced by the dideoxynucleotide chain termination method, using oligonucleotides made to Bluescript vector sequences.

**Northern (RNA) blot analysis.** Five micrograms of total RNA, isolated from a range of different mouse tissues or cell lines by the method of Chirgwin et al. (5), was separated on a 1% agarose gel containing 2.2 M formaldehyde and 20 mM MOPS (3-[N-morpholine]propanesulfonic acid, pH 6.8) and capillary blotted onto a Nitroplus 2000 transfer membrane (MCI, Westboro, Mass.). Filters were prehybridized for a minimum of 4 h in 50% formamide containing 5 $\times$  SSC (1 $\times$

SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10 $\times$  Denhardt's solution, and 50 mM sodium phosphate (pH 5.7) and hybridized overnight in the same solution plus 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg of herring sperm DNA per ml, and 10<sup>6</sup> cpm of <sup>32</sup>P-cDNA, labeled by random priming, of either the 380-bp PCR fragment subclones or full-length cDNAs for PEP, SHP, and human  $\beta$ -actin (the latter a gift from Tim Ley, Washington University) per ml. The final wash of filters was in 0.2 $\times$  SSC-0.1% SDS at 60°C. Autoradiography was carried out for between 1 and 5 days, using Kodak XAR-5 X-ray film. For reuse, filters were stripped with a solution of 0.2 $\times$  SSC-0.1% SDS that had been boiled immediately prior to use and autoradiographed overnight to check for the efficiency of stripping.

**Isolation and sequence analysis of cDNA clones.** Two million recombinant phage from an amplified 70Z/3 pre-B-cell-derived cDNA library (2) were hybridized separately with 380-bp PCR fragments for SHP and PEP, <sup>32</sup>P labeled by the random-primer method. Prehybridization and hybridization of the filters were performed as described above. Phage were tested for size of insert by using a PCR-based approach with oligonucleotides to  $\lambda$ gt11 flanking sequences and oligonucleotides specific for PEP and SHP that had been previously derived by the sequencing of the PCR subclones. Phage that contained putative full-length cDNAs were isolated, and inserts were subcloned into Bluescript KS or SK. The SHP and PEP cDNAs were sequenced by the dideoxynucleotide chain termination method, using oligonucleotide-directed priming of plasmid DNA. Both the SHP (2.1 kb) and PEP (2.7 kb) cDNAs were sequenced in their entirety on both strands.

**In vitro transcription and translation.** Sense transcripts were generated by using T3 polymerase and plasmids linearized 3' of the cDNA coding regions. For mRNA capping, a commercial kit (Stratagene) was used. Similarly, antisense transcripts were generated by using T7 polymerase and plasmids linearized 5' of the cDNAs. All four DNA transcripts were quantitated and checked for integrity by gel analysis. Each transcript was translated in vitro by using a commercial rabbit reticulocyte lysate (Promega), and the products were analyzed on an SDS-8% polyacrylamide gel.

**Bacterial expression of PEP and SHP PTPase domains.** The PTPase domain of SHP (amino acids 235 to 595) was amplified by PCR, using appropriate oligonucleotides specific for SHP but with *Eco*RI and *Hind*III restriction sites incorporated into the 5' and 3' oligonucleotides, respectively. A fragment encoding the SH2 domains of SHP (amino acids 1 to 222) was similarly isolated by PCR but with an *Nco*I restriction site incorporated in the 5' oligonucleotide. The PCR fragments were directionally cloned into pGEX-KG (18, 52). Glutathione S-transferase (GST) fusion proteins were expressed and purified as described by Guan and Dixon (18). The solubility of the fusion proteins was improved dramatically by inducing the bacteria (*Escherichia coli* DH5 $\alpha$ ) at 25°C overnight in isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at 0.1 mM.

The PTPase domain of PEP (amino acids 1 to 289) was also isolated by PCR and cloned into the pGEX expression system but was insoluble. However, a PEP fragment (amino acids 1 to 294) was expressed in a soluble form by using the pMAL bacterial expression system (New England Biolabs) according to the manufacturer's instructions (14). The pMAL-PEP fusion construct was extracted by using a Mini-Bead Beater (Bartlesville, Okla.). Briefly, 5 ml of induced culture was centrifuged, and the pellet was resuspended in 1

ml of 10 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.5)–30 mM NaCl–10 mM  $\beta$ -mercaptoethanol–10 mM dithiothreitol–5 mM EDTA. The bacteria were beat for 1 min, and the insoluble material was removed by centrifugation. The protein concentration was determined by Bradford protein assay (Bio-Rad).

**PTPase activity assays.** (i) **Hydrolysis of *p*-nitrophenyl phosphate.** The purified PTPase domains of SHP and PEP were incubated at room temperature for 10 min with 5  $\mu$ l of 50 mM sodium *p*-nitrophenyl phosphate in a solution containing 62.5 mM morpholineethanesulfonic acid (MES) buffer (pH 6.0), 6.25 mM EDTA, and 12.5 mM dithiothreitol. The reaction was stopped by the addition of 0.2 M NaOH, and the  $A_{410}$  was measured (59).

(ii) **Dephosphorylation of peptide substrates.** Substrates for SHP activity were phosphorylated by taking 40  $\mu$ g of either Raytide (Oncogene Sciences, Manhasset, N.Y.) or poly(Glu:Tyr) (4:1) (Sigma, St. Louis, Mo.) and incubating the samples with 16 U of  $\text{p60}^{\text{src}}$  in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5)–0.1 mM EDTA–0.2% 2-mercaptoethanol–10 mM  $\text{MgCl}_2$ –30  $\mu$ M ATP–40  $\mu$ Ci of [ $\gamma$ - $^{32}\text{P}$ ]ATP in a final volume of 128  $\mu$ l. The reaction mixture was incubated at 30°C for 30 min, and the reaction was stopped by separating the free ATP from the labeled protein over a Sephadex G-25 column (Boehringer Mannheim Corp., Indianapolis, Ind.). Substrates for PEP activity were phosphorylated by taking 30  $\mu$ g of either Raytide, angiotensin, or myelin basic protein and incubating the samples with 16 U of  $\text{p60}^{\text{src}}$  in 50 mM HEPES buffer (pH 7.5)–0.1 mM EDTA–0.2% 2-mercaptoethanol–10 mM  $\text{MgCl}_2$ –30  $\mu$ M ATP–30  $\mu$ Ci of [ $\gamma$ - $^{32}\text{P}$ ]ATP in a final volume of 90  $\mu$ l. The reaction mixture was incubated at 30°C for 60 min, 16 U of  $\text{p60}^{\text{src}}$  was added, and the mixture was incubated at 30°C for 60 min. Phosphorylated peptides were isolated with use of Whatman P81 paper, five washes in 200 ml of 0.5% phosphoric acid, and elution with 1 ml of ammonium bicarbonate. Peptides were lyophilized and resuspended in water. Phosphorylated myelin basic protein was isolated by addition of 0.1 ml of BSA (5 mg/ml) and precipitation with 0.5 ml of 20% trichloroacetic acid.

SHP dephosphorylation was assayed by incubating either purified SHP phosphatase domain or SH2 domain with  $^{32}\text{P}$ -labeled Raytide (6.8  $\mu$ M) or poly(Glu:Tyr) (4:1) (0.35  $\mu$ M) for 10 min in 20 mM imidazole buffer (pH 7.0)–0.2% 2-mercaptoethanol. PEP dephosphorylation was assayed by incubating bacterial lysates containing either the PEP PTPase domain–maltose-binding fusion protein or maltose-binding protein with  $^{32}\text{P}$ -labeled Raytide (5.6  $\mu$ M), angiotensin (7.6  $\mu$ M), or myelin basic protein (0.56  $\mu$ M) for 10 min in 50 mM imidazole (pH 7.0)–0.2% 2-mercaptoethanol. Reactions were performed for a duration of time that had been determined to be at a linear point in a time course experiment. The reactions were stopped by addition of 750  $\mu$ l of charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM  $\text{NaH}_2\text{PO}_4$ , 4% [vol/vol] Norit A). After vortexing and centrifugation, the radioactivity in 500  $\mu$ l of supernatant was determined (55).

**Binding of SHP SH2 domain to phosphotyrosine proteins.** LSTRA cells ( $5 \times 10^7$ ) were lysed in 5 ml of 0.5% Nonidet P-40–50 mM Tris (pH 8.0)–150 mM NaCl–1 mM sodium vanadate–1 mM phenylmethylsulfonyl fluoride–10  $\mu$ g of leupeptin per ml–17.5 TIU of aprotinin, and the nuclei were removed by centrifugation. One-milliliter portions of supernatants were precleared by tumbling with 40  $\mu$ l of 50% glutathione agarose for 1 h at 4°C. The beads were removed by centrifugation, and 10  $\mu$ l of either 50% glutathione agarose prebound with GST or SH2-GST fusion protein was

added in the presence or absence of 50 mM phosphotyrosine. Beads were tumbled for 3 h at 4°C, pelleted by centrifugation, washed once in cold lysis buffer, and boiled for 2 min with 10  $\mu$ l of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. Two microliters was resolved by electrophoresis on an SDS–12% polyacrylamide gel, and proteins were transferred to an Immobilon-P filter (Millipore, Bedford, Mass.) by electrophoresis. The filter was blocked by treatment with 5% BSA–10 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.4)–150 mM NaCl and developed with polyclonal antiphosphotyrosine (provided by Andrey Shaw, Washington University), protein A conjugated to horseradish peroxidase, and enhanced chemical luminescence (Amersham Corp., Arlington Heights, Ill.) as instructed by the manufacturer.

**Immunoprecipitation of SHP.** LSTRA cells ( $2 \times 10^7$ ) were washed twice in methionine-free RPMI 1640 and incubated for 3 h at 37°C in the presence of 300  $\mu$ Ci of [ $^{35}\text{S}$ ]methionine. Cells were pelleted by centrifugation and resuspended in 2 ml of 1% Nonidet P-40–0.4% deoxycholate–50 mM Tris-HCl (pH 8.0)–62.5 mM EDTA–5 mM iodoacetamide–1 mM phenylmethylsulfonyl fluoride–10  $\mu$ g of leupeptin per ml–17.5 TIU of aprotinin. Nuclei were removed by centrifugation, and 1-ml portions were precleared by adding 15  $\mu$ l of preimmune rabbit serum and 20  $\mu$ l of 50% protein A-Sepharose and tumbling for 1 h at 4°C. After removal of the beads by centrifugation, either 5  $\mu$ l of preimmune serum or 5  $\mu$ l of rabbit anti-SHP SH2 domain was added with 20  $\mu$ l of protein A-Sepharose and tumbled overnight (the rabbit antiserum was raised against the SH2 domain that had been expressed as a bacterial fusion protein with GST, proteolytically cleaved, and purified). The beads were pelleted, washed three times with cold lysis buffer containing 0.1% SDS, and boiled with 20  $\mu$ l of SDS–PAGE sample buffer. Samples were resolved on an SDS–8% polyacrylamide gel, and proteins were detected by autofluorography.

**Nucleotide sequence accession number.** The nucleotide sequences for mouse PEP and SHP have been deposited in the GenBank data base under accession numbers M90388 and M90389, respectively.

## RESULTS

**Isolation and tissue expression of cDNA encoding PTPase domains.** cDNA fragments encoding PTPase domains were generated by priming RNA isolated from mouse brain or spleen with a pool of degenerate oligonucleotides derived from conserved PTPase sequences and amplified by PCR. Fragments were subcloned and screened with a cocktail of PTPase-encoding fragments derived from the protochordate *S. plicata* (37). Sequence analysis of 42 subclones revealed 11 that contained the hallmark residues indicative of PTPase domains. Within this group, three novel sequences were found in addition to sequences for CD45 and a fragment that is highly similar to a sequence in human PTP $\delta$  (one amino acid difference) and most likely represents the mouse homolog.

To identify the tissue expression of the novel PTPases, Northern blot analysis was performed. Two PCR fragments, SHP and PEP, hybridized to mRNAs of 2.1 and 2.7 kb, respectively, expressed primarily in hematopoietic tissues. Full-length cDNAs encoding SHP and PEP were isolated by screening a 70Z/3 pre-B-cell library (2). Northern blot analysis using the full-length cDNA probe confirmed previous results obtained by using the PCR fragments as probes (Fig. 1A). PEP is expressed in spleen, thymus, lymph node, and bone marrow and is negligible in other tissues. This pattern

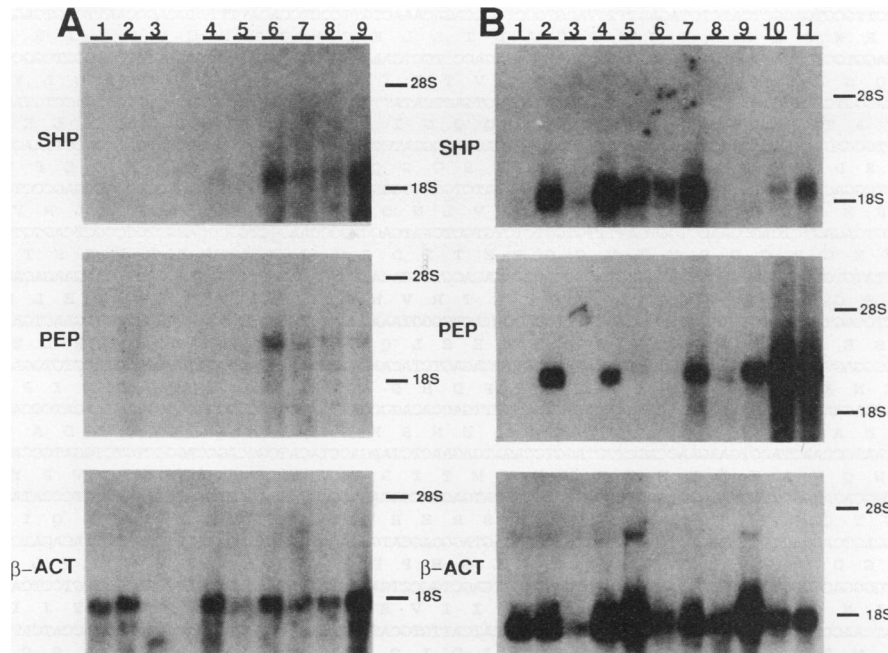


FIG. 1. Northern blot analysis of PTPase expression. (A) Total RNA from a range of different mouse tissues was hybridized with full-length cDNAs for SHP, PEP, and  $\beta$ -actin ( $\beta$ -ACT). Lanes: 1, testes; 2, brain; 3, heart; 4, lung; 5, kidney; 6, spleen; 7, thymus; 8, lymph node; 9, bone marrow. (B) Total RNA from a range of different mouse cell lines was hybridized with full-length cDNAs for SHP, PEP, and  $\beta$ -actin. Lanes: 1, L929 (connective tissue); 2, M1 (myeloblast); 3, J774 (macrophage); 4, WEHI-3 (macrophage); 5, P388D1 (macrophage); 6, S194 (B cell); 7, 70Z/3 (pre-B cell); 8, 2.102 (T-cell hybridoma); 9, BW5147 (thymoma); 10, L3 (CD8<sup>+</sup> T-cell clone); 11, A.E.7 (CD4<sup>+</sup> T-cell clone). Positions of 28S and 18S rRNAs are indicated.

is reminiscent of the hematopoietic cell-specific PTPase, CD45. SHP has a similar pattern of expression but is also weakly expressed in lung and kidney. The cell types expressing SHP and PEP in various tissues are not known; to begin to address this issue, lymphoid and myeloid cell lines were examined (Fig. 1B). SHP is expressed by the myeloblast line M1; the macrophage lines J774, WEHI-3, and P388D1; the B-cell lymphoma S194; and the pre-B-cell line 70Z/3. The expression in T cells is heterogeneous, SHP being expressed by the CD8<sup>+</sup> T-cell clone L3 and the CD4<sup>+</sup> T-cell clone A.E.7 but not by the CD4<sup>+</sup> T-cell hybridoma 2.102 or the thymoma BW5147. In contrast to SHP, PEP is not expressed by some macrophage lines and the B-cell lymphoma but is expressed by the T-cell hybridoma and the thymoma. Neither is expressed by the fibroblastoid line L929.

**SHP sequence.** Sequence analysis of the SHP cDNA revealed a single large open reading frame encoding a 595-amino-acid protein (Fig. 2). Upstream of the predicted initiator methionine (26) at positions 104 to 106 there are stop codons in all three reading frames and no in-frame methionines. There is no identifiable hydrophobic signal sequence; therefore, SHP is predicted to be an intracellular protein. The PTPase domain encompasses residues 247 to 514, leaving an amino-terminal extension and an 80-amino-acid carboxy-terminal tail. The PTPase domain is approximately 35% similar in sequence to other intracellular PTPases (4, 6, 13, 31, 67). The SHP PTPase is not significantly similar in sequence to any other PTPase and thus represents a new subfamily. Within the amino-terminal 214 amino acids is an imperfect tandem repeat of approximately 100 amino acids. The repeated sequence contains approximately 34% identical residues. Comparison of the non-PTPase sequences with entries in the EMBO/GenBank data base revealed significant

similarity of each of the repeat sequences with SH2 domain sequences of c-Src (58), PLC- $\gamma$ 1 (53, 57), and GAP (61, 66) as well as SH2 domain regions in other proteins (25). Thus, SHP contains two SH2 domains linked in tandem at the amino terminus followed by a short spacer region and the PTPase domain. The spacer region and the carboxy-terminal tail do not contain significant homology with other known proteins.

**PEP sequence.** The cDNA for PEP contains a large open reading frame encoding a protein of 802 amino acids (Fig. 3). The initiator methionine (26) is predicted at residues 113 to 115. Upstream of this site is one in-frame methionine at positions 56 to 58, but this residue is followed by two in-frame stop codons. Hydropathy analysis indicates that PEP contains no hydrophobic segments appropriate for signal peptides or transmembrane domains and is thus also predicted to be an intracellular protein. The PTPase domain is located near the predicted amino terminus at residues 23 to 288. Therefore, there is a very large carboxy-terminal extension of 514 amino acids. Comparison of the carboxy-terminal domain with sequences in the EMBO/GenBank data base revealed no significant similarities. However, the carboxy-terminal domain contains an approximately 300-amino-acid proline-, serine-, threonine-, glutamic acid-, and aspartic acid-rich domain from positions 497 to 802. Within this area is an imperfect proline-rich repeat of 14 amino acids located at positions 613 to 626 and 688 to 802. This subdomain contains five separate sequences that are characteristic of proteins with a rapid turnover rate (PEST sequences) and have PEST scores greater than 4 (47). Peptides with PEST scores greater than 0 are indicative of potential rapid degradation (47).

**Expression of PEP and SHP.** To examine whether the

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CCTGAGAGCCCTTCCTGAGGCTCATCTCTAGAGTTTGTACGTGCTGCCCAGACAACTGTTCCCTCCACATTTTCTGCAGOCATTCAGTGAGAACCCC 100
M V R W F H R D L S G F D A E T L L K G R G V F G S F L A R P S 32
AGGATGGTGAAGTGGTTTTCACGGGAOCTCAGCGGGCTGATGCAGAGACCTCTGAAGGGCGGGGAGTCCCTGGGAGCTTCTGGCTGGGCCCCAGCC 200
R K N Q G D F S L S V R V D D Q V T H I R I Q N S G D F Y D L Y G G 66
GCAAGAACCCAGGTGACTTCTCTCAGTCAGGGTGGATGATCAGGTGACTCATATTGGATCCAGAACTCAGGGGACTTCTATGACCTGTACGGAGG 300
E K F A T L T E L V E Y T L Q Q G I L Q D R D G T I I H L K Y P 99
GGAGAAGTTTGCAGCCTGACAGAGCTGGTGGAGTATTACACCGCAGCAGCGGCATCTGCCAGGACGAGATGCCACCATCATCCACTTAACTACCCA 400
L N C S D P T S E R W Y H G H I S G G Q A E S L L Q A K G E F W T 132
CTGAACCTGCTCGGACCCCCAGCTGAGAGGTGGTACACCGGCCACATATCTGGAGGGCAGGGGAGTCACTGCTGCAGGCCAAGGGCGAGCCCTGGACAT 500
F L V R E S L S Q P G D F V L S V L N D Q R K A G P G S F L R V T H 166
TTCTTGTGGTGAAGTCTCAGCCAACTGGTGAATTTTGTGCTCTCTGCTCAATGACAGCCCAAGGCTGGCCCGAGGTTCGCCGCTCAGGGTCACTCA 600
I K V M C E G G R I T V G G S E T F D S L T D L V E H F K K T G I 199
TATCAAGGTTATGTGTGAGGGTGGACGCTATCTGTGGGTGGCTCAGAGAGCTTTGACAGCCTCAGAGCTGGTGGAGCACTTCAAGAAGCAGGGATT 700
E R A S G A F V Y L R Q P Y Y A T R V N A A D I E N R V L E L N K 232
GAGGAGGCTCGGGTGGCTTGTCTAAGTGGCGAGCTTACTAAGTGGTAAAGCAGCTGACATTCAGAACTGGGCTCTTGGAACTCAACAAGA 800
K Q E S E D T A K A G F W E [E F E S L Q K Q E V K N L H Q R L E G Q 266
AGCAGGAGCTCGGAGGACAGCCAGGCTGGCTTCTGGGAGGAGTTTGAGAGCTCTACAAAGCAGGAGGTAAGAATCTACACCAAGCTCTGGAAGGGCC 900
R P E N K S K N R Y K N I L P F D H S R V I L Q G R D S N I P G S 299
GGCGCCAGAGAACAGAGCAAGACCCCTACAAGAACATCTCTCCCTTTGACAGCAGCGAGTGATCCCTGCAGGAGCGTGACAGTAACATCCAGGCTCT 1000
D Y I N A N Y V K N Q L L G P D E N S K T Y I A S Q G C L D A T V 332
GACTACATCAATGCCAACTACGTGAAGAACCAGCTGCTAGGTCCAGATGAGAATCTAAGACCTACATCGCCAGCCAGGGCTGTCTGGATGCCACAGTCA 1100
N D F W Q M A W Q E N T R V I V M T T R E V E K G R N K C V P Y W P 366
ATGACTTCTGGCAGATGGCTTGGCAGGAGCACTGGTGTCTATGCTCATGACTACACAGAGGTTGGAGAAAGGGCCGAACAATGTGTCCCATCTAGTGGC 1200
E V G T Q R V Y G L Y S V T N S R E H D T A E Y K L R T L Q I S P 399
CGAGGTGGGCACTCAGCGTCTCTAGGTCTCTACTCTGTGACCAACAGTATAGGAGCATGACACAGCAGAAATACAACTCGGACATTTAGCATCTCCCA 1300
L D N G D L V R E I W H Y Q Y L S W P D H G V P S E P G G V L S F 432
CTAGACAATGGGCACTGTGTGGGAGATATGGCACTACCACTGAGCTGAGCTGGCCATGACCATGGGGTTCGCCAGTGAGCTGGGGGTCTCTCTCATCTTC 1400
L D Q I N Q R Q E S L P H A G P I I V H C S A G I G R T G T I V I 466
TGGATCAGATCAACCGAGGACAGGAAGTTTGCTCATGCGAGGGCCCATCATTTGTGCATTGCGAGCGTGGCATGGGCGCAGGGCCACCATCATCGTCAT 1500
D M L M E S I S T K G L D C D I D I Q K T I Q M V R A Q R S G M V 499
TGATATGCTTATGGAAGCATCTCCACCAAGGGGCTAGACTGTGACATTCATTCAGAAAGCATCCAGATGGTACGAGCAGCAGCGCTCGGCACTGGTG 1600
Q T E A Q Y K F I Y V A I A Q [F I E T T K K K L E I I Q S Q K G Q 532
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E S E Y G N I T Y P P A V R S A H A K A S R T S S K H K E E V Y E N 566
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V H S K S K K E E K V K K Q R S A D K E K N K G S L K R K * 595
CGTGATAGCAAGAGCAAGAGGAGGAGAAAGTAAAGAGCAGCGGTGGCAGACAGGAGAGAAACAAGGTTCTCTCAAGAGGAAGTGTATCTGGGCAT 1900
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GGACCACTGTAAAAA 2122

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FIG. 2. Nucleotide and predicted amino acid sequences of the SHP cDNA clone. The SH2 domains are shaded, and the PTPase domain is indicated by half-brackets. The polyadenylation site is underlined. The single-letter amino acid code is used.

predicted proteins encoded by the cDNAs could be expressed, the cDNAs were ligated into a vector containing T7 and T3 polymerase sites, and in vitro transcription and translation were performed (Fig. 4). The cDNA sequence for SHP predicts a protein with an  $M_r$  of 67,559, and the molecular weight of in vitro translation product is in good agreement with this value. The molecular weight of the predicted protein for PEP is 89,714. Two major products with  $M_r$ s of 89,000 and 97,000 were obtained. It is possible that the high-molecular-weight product is a form modified by the rabbit reticulocyte lysate. However, because of the large number of minor lower-molecular-weight products, it is probable that there is either proteolytic degradation or premature termination of the translate and that the high-molecular-weight form is the intact PEP protein. It is possible that PEP migrates anomalously on SDS-PAGE because of the high proline and acidic amino acid content found in the carboxy-terminal domain. Aberrant migration on SDS-PAGE has been reported for two other PEST-containing proteins, Vp1 and Bmi1 (40, 65).

**Enzymatic activity.** The portions of SHP and PEP encoding the PTPase domain and the SH2 domain of SHP were engineered into bacterial expression vectors. The purified protein for the SHP PTPase domain, but not the SH2 domain, contained PTPase activity when assayed by dephosphorylation of *p*-nitrophenyl phosphate (data not shown). To examine further the phosphatase activity of SHP, the PTPase domain was assayed with phosphorylated Raytide

and poly(Glu:Tyr) (Fig. 5D and E). PTPase activity with these substrates was readily detected for the PTPase domain but not for the SH2 domain.

The PEP PTPase domain became insoluble when proteolytically digested to remove the fusion protein partner. However, the PEP PTPase-maltose-binding fusion protein, which had very weak activity when assayed against *p*-nitrophenyl phosphate, had very good activity when assayed with phosphorylated Raytide (Fig. 5A). The control of bacterially expressed maltose-binding protein had little, if any, activity toward this substrate. In addition, the PEP PTPase fusion protein dephosphorylated  $^{32}$ P-labeled myelin basic protein and angiotensin (Fig. 5B and C).

**SHP SH2 domains interact with a subset of phosphotyrosine proteins.** The SH2 domains of SHP were incubated with lysate of the lymphoma line LSTRA. Western immunoblot analysis with an antiphosphotyrosine antibody revealed that a subset of phosphotyrosine could bind directly to the SH2 domains and could be specifically blocked by incubation with phosphotyrosine (Fig. 6A). Most notable was the interaction of a 15-kDa protein. The 15-kDa protein did not appear to be a major constituent of the total phosphoprotein; therefore, there appears to be a preferred interaction between the SHP SH2 domain and this protein. To determine whether SHP is expressed by LSTRA cells, cells were metabolically labeled and SHP was immunoprecipitated. A specific 67-kDa band was revealed by SDS-PAGE (Fig. 6B). Therefore, it is possible that the 15-kDa protein is a physio-

FIG. 3. Nucleotide and predicted amino acid sequences of the PEP cDNA clone. The PTPase domain is indicated by half-brackets. The five sequences that match the consensus for a PEST sequence (47) are overlined, and the imperfect proline-rich repeat is shaded. The polyadenylation site is underlined. The single-letter amino acid code is used.

The novel structures of SHP and PEP and the 35% sequence similarity of the PTPase domains to other PTPases indicates that these two molecules represent additional PTPase intracellular subfamilies. The identification and characterization of a variety of mammalian PTPases highlight their diversity in structure, and therefore function, and accentuate the importance of PTPases in regulating tyrosine phosphory-

## DISCUSSION

**Diversity and patterns of expression of intracellular PTPases.** This study has identified two intracellular PTPases, SHP and PEP, that are likely to have important regulatory roles within leukocytes. The patterns of expression for SHP and PEP mRNAs indicate that they are both synthesized primarily in hematopoietic tissues, including bone marrow,

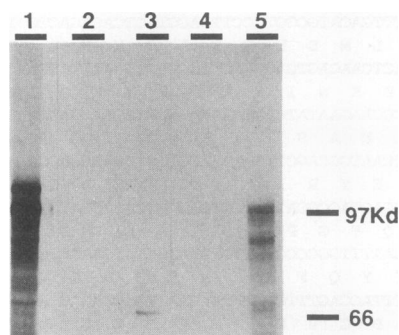


FIG. 4. Expression of SHP and PEP proteins. cDNAs for SHP and PEP were transcribed and translated in vitro to produce antisense (lanes 2 and 4) and sense (lanes 3 and 5) transcripts. Products were analyzed on an SDS-8% polyacrylamide gel. Lanes: 1, brome mosaic virus RNA translated in vitro to produce proteins with  $M_r$ s of 110,000 and 97,000; 2, SHP antisense product; 3, SHP sense product; 4, PEP antisense product; 5, PEP sense product.

lation, an aspect not previously appreciated in studies focused on PTKases. The intracellular PTPases currently characterized can be divided into five subfamilies (Fig. 7).

**SH2 domain-mediated signal transduction.** The deduced amino acid sequence for SHP contains a carboxy-terminal PTPase domain that has been demonstrated in vitro to possess catalytic activity toward phosphotyrosine in the context of two artificial substrates. At the amino terminus of the SHP protein, two tandem domains that bear the hallmarks of previously described SH2 domains are found. The SH2 domains of SHP are as different from each other as they

are from the SH2 domains of other proteins. SHP is similar to PLC- $\gamma$  (53, 57), GAP (61, 66), and P85 $\alpha$  and P85 $\beta$  (43) in possessing two SH2 domains. Unlike other SH2 domain-containing proteins, SHP does not contain a 45-amino-acid SH3 domain which has been suggested to be involved in cytoskeletal association (25).

It is possible that the SH2 domain repeat may allow for synergistic binding to the same substrate. Alternatively, each SH2 domain might bind to distinct substrates. The demonstration that SHP SH2 domains bind a 15-kDa protein suggests that SHP may interact with this protein via at least one SH2 domain. It is interesting to note that a 15-kDa fatty acid-binding phosphotyrosine protein expressed by adipocytes interacts with a 60-kDa PTPase (30). Whether it is a related 15-kDa protein that is expressed by LSTRA cells and binds the SH2 domain of SHP remains to be determined.

Recent work suggests that several tyrosine kinase receptors initiate signal transduction pathways via the formation of SH2 domain-mediated complexes (reviewed in reference 3). Activation of tyrosine kinase receptors leads to an association with intracellular signaling molecules via phosphotyrosine on the receptor (7, 9, 21, 23, 24, 32–34, 41). For example, the activated platelet-derived growth factor receptor binds to PLC $\gamma$ -1 (41), GAP (21, 24), and the P85 subunit of the phosphatidylinositol 3-kinase (7, 9, 23). Furthermore, in vitro studies have indicated that the SH2 domain within the individual intracellular signaling molecules mediates binding to the activated tyrosine kinase receptors (1, 39, 42). As tyrosine phosphorylation is a reversible biochemical event, there must presumably be a PTPase that regulates the formation of SH2 domain-mediated complexes. By possessing two SH2 domains, SHP may be one of a family of

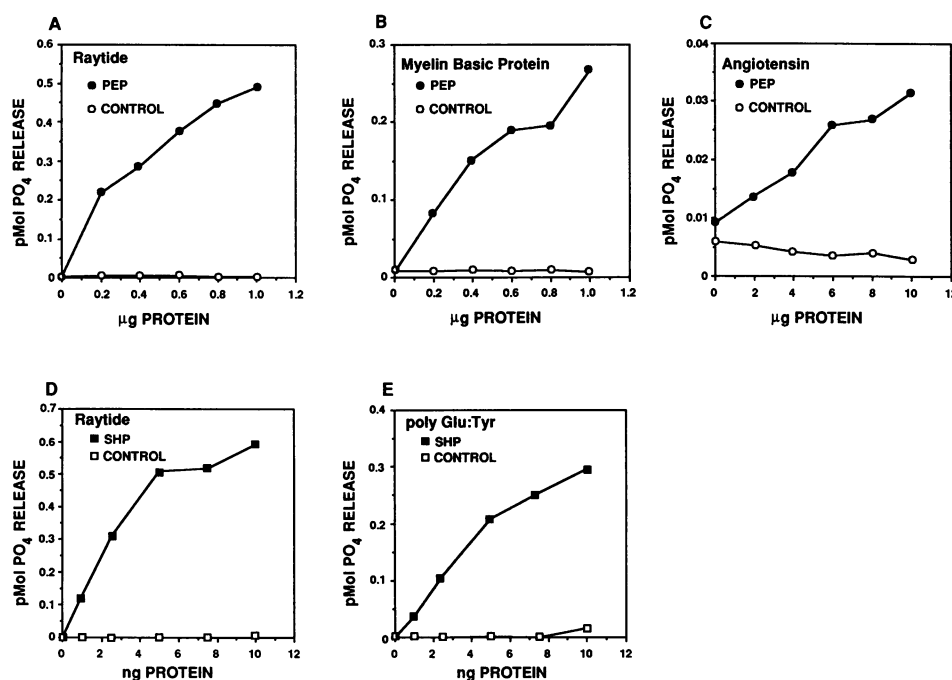


FIG. 5. PEP and SHP PTPase activities. The PTPase domain of PEP was expressed as a fusion protein and used in crude bacterial lysates. To control for endogenous phosphatase activity, maltose-binding protein was expressed in bacteria and used as a crude bacterial lysate. The PTPase and SH2 domains of SHP were expressed in bacteria and purified by affinity chromatography. Dephosphorylation was assayed by incubating PEP and SHP PTPases with  $^{32}$ P-labeled proteins as indicated.

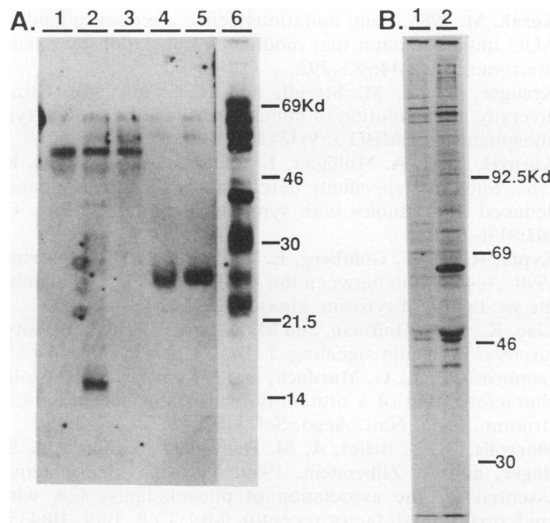


FIG. 6. Interaction of the SHP SH2 domain with phosphotyrosine proteins and immunoprecipitation of SHP from LSTRA cells. (A) Phosphotyrosine immunoblot of LSTRA proteins that bound to SH2-GST. Lanes: 1, SH2-GST without mixing with LSTRA cell lysates; 2, SH2-GST mixed with LSTRA cell lysates; 3, SH2-GST mixed with LSTRA cell lysates in the presence of phosphotyrosine; 4, GST without mixing with LSTRA cell lysates; 5, GST mixed with LSTRA cell lysates; 6, LSTRA cell lysate. The 52-kDa SH2-GST fusion protein and 26-kDa GST are visualized as background bands. (B) Immunoprecipitation of [<sup>35</sup>S]methionine-labeled LSTRA cells. Lanes: 1, preimmune serum; 2, anti-SHP SH2 domain.

PTPases that act as negative regulators of SH2 domain-directed complexes.

Matsuda et al. (35) have proposed a model whereby the SH2 domain of the nonreceptor PTKases binds through an intramolecular association to a negative regulatory carboxy-terminal tyrosine phosphorylation site. There are three tyrosine residues in the carboxy terminus of SHP which, if phosphorylated, could also contribute to an intramolecular regulatory mechanism.

**The non-PTPase domain of PEP.** The most striking feature of the PEP protein is the presence of a large 500-amino-acid carboxy-terminal domain that has no significant sequence similarities to any known protein. However, within the carboxy-terminal domain there is a sequence of 300 amino acids that is enriched for proline (12%), serine/threonine (26%), and glutamate/aspartate (11%). A preponderance of these amino acids (PEST sequences) is a characteristic of proteins that are rapidly degraded within eukaryotic cells (47). As PEP possesses five such consensus PEST sequences, it might be expected to have an extremely short intracellular half-life. PEST sequences have been reported in a number of proteins that are located in the nucleus, including c-Fos, c-Myc, p53, Bmi1, and Vp1 (40, 47, 65). Interestingly, there is a basic sequence (PVKRTK) at positions 446 to 451 in PEP that satisfies the criteria for one class of nuclear localization signal (8, 11). In support of this notion, there is a putative casein kinase II site (SSEE) at positions 497 to 550. Casein kinase II sites have been located close to many confirmed nuclear localization signal sequences found in other proteins, and phosphorylation at these sites has been implicated in the rate of nuclear transport (46). It is possible, therefore, that PEP is a PTPase with a short half-life that localizes to the nucleus. Interestingly,

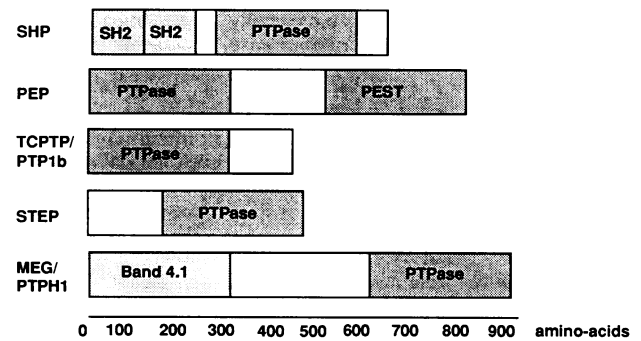


FIG. 7. Schematic diagram of mammalian intracellular PTPases.

PEP also contains three potential sites for phosphorylation by the p34<sup>cdc2</sup> kinase, raising the possibility that PEP is phosphorylated in a cell cycle-dependent manner. These sites are at positions 667 to 671 (FTPSK), 735 to 741 (RTPGK), and 760 to 764 (SSPSK), and they conform to the consensus sequence for p34<sup>cdc2</sup> kinase, XS/TPXK/R (45, 51). Whether any or all of these sequences function as found in other proteins remains to be determined. Only two protein tyrosine kinases, c-Abl and c-Fer, have so far been localized to the nucleus (20, 64).

During the preparation of this report, Shen et al. (50) published a report describing the isolation of a PTPase, PTP 1C, from a human breast carcinoma cell line. A comparison of the sequences of PTP 1C and SHP reveals 95% sequence identity, indicating that they are species homologs. One notable difference between the two sequences is the insertion of an adenosine residue at position 1868 in the mouse sequence, which causes a frameshift and change in the stop codon.

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